

Molecular Cloning and Characteristics of Catalase cDNA from Chinese Soft-shelled Turtle (*Pelodiscus sinensis*)

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Abstract A catalase cDNA was cloned from the liver of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) using reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers. Both 3'- and 5'-untranslated regions were isolated by the rapid amplification of cDNA ends method (RACE). Analysis of nucleotide sequence revealed that the catalase cDNA clone consisted of 2173 bp with an open reading frame of 1587 bp encoding a protein of 528 amino acids. The calculated molecular mass of the mature protein is 59.8 kDa with an estimated pI of 6.84. The peroxisomal targeting signal SNL at the C-terminal and two putative N-glycosylation sites NLSV and NVSQ were found in the catalase. Sequence comparison showed that this catalase, deduced by the amino acid sequence, had high similarity and identity with those of vertebrates recorded in GenBank. Four functional domains and conserved amino acids responsible for binding heme and NADPH including four essential residues were observed. The 3-D homology model of the turtle catalase was predicted by SwissModel based on the relative domains of bovine catalase structure (PDB ID: 3rgp). The mRNA expression and enzyme activities in liver, brain, spleen, kidney, heart, gut, lung and muscle were investigated, and the results showed that the mRNA and enzyme activities of catalase in these tissues were species-specific.

Keywords sequence comparison, 3-D homology model, expression, enzyme activity, *Pelodiscus sinensis*

1. Introduction

Catalase (EC 1.11.1.6) is a crucial antioxidant enzyme that decomposes millions of hydrogen peroxide molecules into water and molecular oxygen without production of free radicals (Nicholls *et al.*, 2000; Young and Woodside, 2001). This protein is a homotetramer with one heme per subunit (Goyal and Basak, 2010; Zámocký *et al.*, 2008; Zámocký and Koller, 1999). Enzymes binding an additional NADPH per subunit are included into the small-subunit group, and, in contrast, the large-subunit enzymes do not bind NADPH but have extended carboxyl ends forming the flavodoxin-like structure (Fita and Rossmann, 1985; Nicholls *et al.*, 2000; Putnam *et al.*, 2000). Catalases are largely located within peroxisomes with peroxisomal targeting signal (PTS) at the C-terminus

(Zámocký and Koller, 1999).

Lack or reduction of catalase activity leads to accumulation of reactive oxygen species (ROS) and consequently oxidative stress (Lushchak, 2011; Zámocký *et al.*, 2008). Macromolecules, including lipids, proteins and nucleic acids, may be damaged (Goyal and Basak, 2010; Valko *et al.*, 2007). Some diseases are related to low activity of catalase (Valko *et al.*, 2007), and genetic mutation causes acatalasemia or hypocatalasemia (Ogata *et al.*, 2008; Zámocký *et al.*, 2008). Overexpression of catalase can also have adverse physiological effects (Zámocký *et al.*, 2008). High enzyme activity may disturb the physiological process related to signaling of hydrogen peroxide (Veal *et al.*, 2007).

Environmental factors and xenobiotics may induce oxidative stress and change the mRNA expression or activity of antioxidant enzymes, including catalase (Hermes-Lima *et al.*, 2001; Lushchak, 2011). Moreover, catalase is one main index of the antioxidant defense (Filho *et al.*, 1993). Many studies have been carried out to investigate the adaptive strategies of antioxidant enzymes to environmental stress, but have centered primarily at the level of protein or enzyme function (Hermes-Lima *et al.*,

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2001; Storey, 1996). The genetic studies provide a huge range of opportunities to examine adaptive responses and understand the mechanisms of gene regulation when animals are confronted with stressful conditions (Storey, 2004).

The Chinese soft-shelled turtle (*Pelodiscus sinensis*), with its great economic importance, is a candidate to study these adaptive responses or strategies of antioxidant enzymes in reptiles, so as to provide basic data for turtle culture. Although genes coding for catalases have been sequenced for more than 120 organisms including fish, amphibians, birds and mammals, and fourteen 3-D structures have been solved (Zámocký *et al.*, 2008), no complete cDNA sequence of a reptilian catalase has yet been studied. This paper provides our results from the cloning of the full cDNA sequence of catalase in the Chinese soft-shelled turtle by RT-PCR and RACE, and investigation of molecular characteristics of the turtle catalase, and its enzyme activity and mRNA expression in the tissues of *P. sinensis* adults.

2. Materials and Methods

2.1 Animals Eight adult turtles (706.75 ± 21.24 g) from Baoding, Hebei, China were housed at a density of 4 turtles in $140\text{ cm} \times 60\text{ cm} \times 70\text{ cm}$ (length $\times$ width $\times$ height) tank with naturally diurnal cycle and fed with standard diets for three weeks before sampling. Water temperature was maintained at $27\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ with one-third water being changed once per day. The samples from these turtles' tissues, including liver, brain, spleen, kidney, heart, gut, lung and muscle, were dissected, snap-frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. The experimental procedures were performed on the standards of the China Council on Animal Care.

2.2 Amplification and cloning of an internal cDNA fragment PCR amplification was done using degenerate primers (listed in Table 1) designed based on the cDNA sequence alignment of human (*Homo sapiens*; GenBank accession number NM_001752.3), mouse (*Mus musculus*; NM_009804.2), red junglefowl (*Gallus gallus*; NM_001031215.1 and AJ719360.1), Africa clawed frog (*Xenopus laevis*; NM_001095194.1 and NM_001087075.1), western clawed frog (*Silurana tropicalis*; NM_001016716.2) and zebrafish (*Danio rerio*; NM_130912.1) catalases.

Hepatic RNA was extracted by TRIzol[®] (#15596-026, Invitrogen, USA) and cDNA was synthesized by PrimeScript II 1st Strand cDNA Synthesis Kit (#D6210A, TAKARA, Japan). PCR was done in a 20 μl reaction

containing 10 μl Premix Taq[®] Version 2.0 (#D332C, TAKARA, Japan), 0.4 μl each primer (20 μM), 7.2 μl ddH₂O and 2 μl cDNA under the following conditions: at $94\text{ }^{\circ}\text{C}$ for 5 min (1 cycle); at $94\text{ }^{\circ}\text{C}$ for 30 sec, at $55\text{ }^{\circ}\text{C}$ for 30 sec, at $72\text{ }^{\circ}\text{C}$ for 1 min (30 cycles); and at $72\text{ }^{\circ}\text{C}$ for 5 min (1 cycle). The amplified fragment was purified and cloned into the pMD19-T Simple Vector (#D104C, TAKARA, Japan) using *Trans5a* Chemically Competent Cell (#CD201-02, TransGen Biotech, China) following the manufacturer's recommendations. Five clones were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The amplified fragments by three pairs of degenerate primers were spliced together.

2.3 Rapid amplification of cDNA ends (RACE) Total RNA was isolated from liver using NucleoSpin[®] RNA II (#740955.250, MACHEREY-NAGEL, Germany) and its integrity was confirmed by 1% gel agarose gel electrophoresis. Approximately 5 μg of total RNA were used for synthesizing cDNA for 3'-RACE and 5'-RACE, respectively following the manufacturer's recommendations of SMARTer[™] RACE cDNA Amplification Kit (#634923, Clontech, USA). The primers for 3'-RACE and 5'-RACE were designed on the spliced sequence obtained in 2.2 and listed in Table 1.

To amplify the 3'-end of the cDNA, 5 μl UPM (5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3', 0.4 μM and 5'-CTAATACGACTCACT A TAGGGC-3', 2 μM) and 1 μl 3P1 (10 μM) were used in 20 μl reaction which contained 0.2 μl Phusion[®] High-Fidelity PCR Kit (#E0553L, NEB, UK), 4 μl 5X Phusion HF buffer, 0.4 μl dNTP Mixture (10 mM, respectively), 0.5 μl cDNA and 8.9 μl ddH₂O. Touchdown PCR was used under the conditions: at $98\text{ }^{\circ}\text{C}$ for 1 min (1 cycle); at $98\text{ }^{\circ}\text{C}$ for 10 sec, annealing for 30 sec decreased by $1\text{ }^{\circ}\text{C}$ per cycle from $72\text{ }^{\circ}\text{C}$, at $72\text{ }^{\circ}\text{C}$ for 1 min (11 cycles); at $98\text{ }^{\circ}\text{C}$ for 10 sec, at $61\text{ }^{\circ}\text{C}$ for 30 sec, at $72\text{ }^{\circ}\text{C}$ for 1 min (24 cycles); and at $72\text{ }^{\circ}\text{C}$ for 5 min (1 cycle) according to the protocol of Mattick and Korbie (Mattick and Korbie, 2008).

To obtain the 5'-end of the cDNA, UPM and 5P1 (10 μM) were utilized under the same conditions as 3'-RACE PCR, and then the amplified products diluted in ratio of 1:49 were used as template for nested PCR and NUP (5'-AAGCAGTGGTATCAACGCAGAGT-3', 10 μM) and 5P2 (10 μM) as primers. The 20 μl reaction comprised of 1 μl NUP, 1 μl 5P2 and 12.9 μl ddH₂O, and the rest was the same as the former reaction. PCR program was identical to the former except for the cycling program: at $98\text{ }^{\circ}\text{C}$ for 10 sec, annealing for 30 sec decreased by $1.5\text{ }^{\circ}\text{C}$ per 2 cycles from $72\text{ }^{\circ}\text{C}$, at $72\text{ }^{\circ}\text{C}$ for 1 min (12 cycles);

and at 98 °C for 10 sec, at 63 °C for 30 sec, and 72 °C for 1 min (23 cycles).

The RACE products were cloned using *pEASY*TM-Blunt Simple Cloning Kit (#CB111-02, TransGen Biotech, China) and *TransI-T1* Phage Resistant Chemically Competent Cell (#CD501-02, TransGen Biotech, China). Five clones were sequenced, and the amplified fragments by RACE and internal fragment were spliced together to get the full cDNA.

2.4 Sequence analysis and phylogenetic analysis

Multiple alignment of nucleotide sequences and splicing of amplified fragments were performed by Bioedit v 7.0.5 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The amino acid sequence was deduced with on-line program Translate (<http://web.expasy.org/translate/>). Theoretical molecular weight and isoelectric point were computed by Compute PI/WM (http://web.expasy.org/compute_pi/). Functional pattern was analyzed by on-line software PROSITE (<http://www.expasy.org/prosite>). The predicted tertiary structure was established using the SWISS-MODEL website (Arnold *et al.*, 2006; Benkert *et al.*, 2011; Guex and Peitsch, 1997; Schwede *et al.*, 2003), and potential glycosylation sites were forecasted by NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

The similarity and identity of the turtle catalase with vertebrate catalases were compared with the complete sequences recorded in GenBank and analyzed with Matrix Global Alignment tool (MatGAT) v 2.0.3. A rooted phylogenetic tree of those catalases was determined using the neighbour-joining distance method by program MEGA v 5.0 (Tamura *et al.*, 2011). The relative importance of branching order was evaluated by the bootstrapping method (1000 replications). Catalases of species selected from fish, amphibians, birds and mammals were aligned with turtle catalase on amino acid level by ClustalW2 (<http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalw2>).

2.5 Semiquantitative PCR to measure mRNA expression pattern in different tissues

Specific primers BCF (20 μM) and BCR (20 μM) were designed, and β-actin was used as a reference gene with primers BAF (20 μM) and BAR (20 μM). Total RNA from eight tissues was extracted by Kit NucleoSpin[®] RNA II, and cDNA was synthesized by PrimeScript II 1st Strand cDNA Synthesis Kit. PCR conditions were identical with those in 2.2, except that the annealing temperature was kept at 59 °C.

2.6 Enzyme activities in tissues Homogenates were prepared with 0.75% sodium chloride by mechanical

method. Specific activity of catalase was measured by Kit (#A003, Nanjing Jiancheng Bioengineering Institute, China), and one unit was defined as a decrease of μmol hydrogen peroxide per minute and total protein. Total protein concentrations in tissues were determined by Coomassie brilliant blue method using bovine serum albumin as a reference protein (#A045-2, Nanjing Jiancheng Bioengineering Institute, China).

2.7 Statistical analysis All data were analyzed using the statistical package of statistical product and service solutions (SPSS) (version 11.0; SPSS Inc., USA). The differences among the catalase activities of the eight tissues were compared with one-way ANOVA followed by least significant difference post hoc test. Values are expressed as mean ± S. D.

3. Results

We successfully obtained the cDNA sequence of catalase in turtle, as shown in Figure 1. The full length is 2173 bp with 33 bp of 5'-untranslated region (5'-UTR) and 553 bp of 3'-UTR. The open reading frame (ORF) is 1587 bp, and the deduced protein contains 528 amino acids. The nucleotide sequence and amino acid sequence have been submitted to the National Center for Biotechnology Information (NCBI) of China, and the GenBank accession number for the nucleotide sequence is JX452102. In 3'-UTR, no CA repeat sequence was found but a poly (A) signal (AATTAAA) just upstream 90 bp of poly (A) tail was identified. PST1 at C-terminus is Ser-Asn-Leu, and no similar motif as Ser-His-Ile in mouse and Ser-His-Met in rat is located near C-terminus of PST1 (Purdue and Lazarow, 1996). Asn-Leu-Ser-Val (residues 244 to 247) and Asn-Val-Ser-Gln (residues 439 to 443) were predicted to be the N-glycosylation sites.

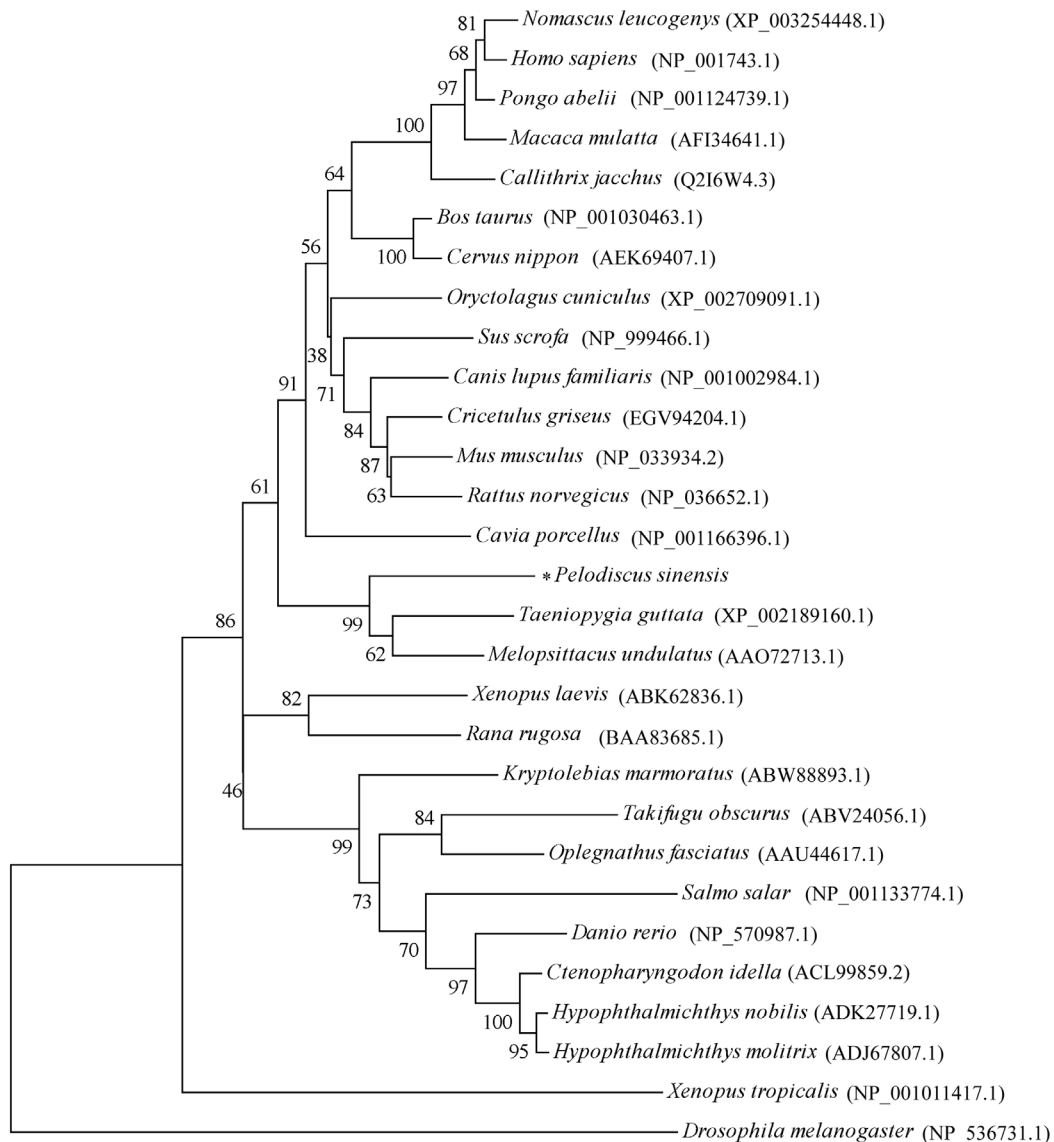
The predicted size of the turtle catalase polypeptide is 59.80 kDa and isoelectric point is 6.84. Multiple comparison between the deduced amino acid sequences of turtle catalase with complete protein sequences of vertebrates recorded in GenBank revealed that the turtle catalase displayed higher sequence similarity to the catalases of two birds, zebra finch (*Taeniopygia guttata*) or budgerigar (*Melopsittacus undulatus*) (data not shown). The same catalases were also applied to reconstruct a neighbour-joining tree with catalase of fruitfly as the outgroup. As shown in Figure 2, the turtle catalase was placed in a cluster with those of zebra finch and budgerigar, and then it was found more related to mammal catalases than the amphibian catalases and fish catalases.

GCGCTTCCAGCGCCGGGGTCGCTAGCCACGCA ATG GCC GGC AAT CGG GGT CCC GCC GAG GAC	63
M A G N R G P A E D	10
CAG CTG AAG CTA TGG CAG GAT CAG CGG GGC TTC CAG AAA CCG GAT GCC TTG ACC ACT GGA	123
Q L K L W Q D Q R G F Q K P D A L T T G	30
GCT GGG ATC TCA GTA GGT GAT AAA CTT AAC ATC ATG ACA GTT GGG CCA AGG GGC CCT CTT	183
A G I S V G D K L N I M T V G P R G P L	50
CTT GTT CAG GAT GTT GTT TTC ACT GAT GAG ATG GCT CAT TTT GAC AGA GAG AGG ATT CCT	243
L V Q D V V F T D E M A H F D R E R I P	70
GAG AGA GTT GTG CAT GCC AAA GGA GCA GGA GCT TTT GGC TAT TTT GAA GTG ACT CAT GAT	303
E R V V H A K G A G A F G Y F E V T H D	90
ATC ACC AAA TAT TGT AAG GCG AAG GTA TTT GAG CAC ATT GGA AAA AGA ACG CCA ATA GCT	363
I T K Y C K A K V F E H I G K R T P I A	110
ATT CGA TTC TCC ACT GTT GCT GGG GAG TCT GGT TCA GCT GAC ACA GTT CGT GAC CCT CGA	423
I R F S T V A G E S G S A D T V R D P R	130
GGC TTT GCC ATG AAA TTC TAC ACA GAA GAA GGT ATC TGG GAT CTT GTT GGC AAC AAC ACT	483
G F A M K F Y T E E G I W D L V G N N T	150
CCC ATC TTC TTT ATC CGG GAT GCC ATG CTG TTT CCA TCC TTT ATA CAT ACC CAA AAG AGG	543
P I F F I R D A M L F P S F I H T Q K R	170
AAC CCT CAG ACT CAC CTG AAG GAT CCA GAC ATG ATG TGG GAC TTC TGG AGC CTT CGC CCT	603
N P Q T H L K D P D M M W D F W S L R P	190
GAA TCT CTG CAC CAG GTA TCT TTC TTG TTC AGT GAT CGT GGT ATT CCA GAT GGA CAC CGC	663
E S L H Q V S F L F S D R G I P D G H R	210
CAT ATG AAT GGA TAC GGA TCC CAT ACT TTT AAA CTG ATT AAT GCA AAT GGC AAA GCT GTT	723
H M N G Y G S H T F K L I N A N G K A V	230
TAC TGC AAA TTT CAT GCA AAG ACT GAT CAG GGC ATC AAA AAC CTT TCT GTC GAA GAA TCA	783
Y C K F H A K T D Q G I K <u>N L S V</u> E E S	250
GGA AGA TTG GCT TCC ACT GAT CCT GAC TAT GCA ATA CGT GAC CTT TAC AAC GCC ATT GCC	843
G R L A S T D P D Y A I R D L Y N A I A	270
AGT GGA AAT AGT CCA TCC TGG ACT TTT TAT ATC CAG GTT ATG ACG TTT GAA GAA GCT GAG	903
S G N S P S W T F Y I Q V M T F E E A E	290
AAA TTC CCA TTT AAT CCT TTT GAT CTA ACT AAG GTT TGG CCT CAT AAA GAC CAT CCT CTA	963
K F P F N P F D L T K V W P H K D H P L	310
ATT CCT GTG GGC AAA CTT GTC TTG AAC AGG AAT CCT GTC AAT TAT TTT GCT GAG ATA GAA	1023
I P V G K L V L N R N P V N Y F A E I E	330
CAA ATA GCC TTT GAT CCA AGC AAC ATG CCA CCA GGT ATC GAA CCT AGC CCT GAT AAA ATG	1083
Q I A F D P S N M P P G I E P S P D K M	350
TTA CAG GGT CGT CTT TTC TCA TAT CCT GAT ACT CAC CGA CAC CGT TTA GGA CCC AAC TAC	1143
L Q G R L F S Y P D T H R H R L G P N Y	370
CTA CAA ATA CCT GTG AAT TGT CCC TAC AGG GCT CGT GTG GCC AAC TAT CAG AGA GAT GGA	1203
L Q I P V N C P Y R A R V A N Y Q R D G	390
CCT ATG TGT GTG TCT GAC AAC CAA GGT GGT GCC CCA AAT TAT TAC CCA AAC AGT TTT GCT	1263
P M C V S D N Q G G A P N Y Y P N S F A	410
GGC CCT GAA GAT CAG CCC AAT TGG AAA GAG AGC CGC ATG TCT GTT TCA GGT GAT GTG CAG	1323
G P E D Q P N W K E S R M S V S G D V Q	430
CGC TTC AAT AGT GCA AAT GAG GAT AAT GTG TCT CAG GTG AGA GTG TTC TAT ACC AAA GTG	1383
R F N S A N E D <u>N V S Q</u> V R V F Y T K V	450
CTG AAA GAA GAT GAA CGC CAA AGA CTG TGT GAG AAC ATT GCT AGC CAT CCT AAA GAT GCT	1443
L K E D E R Q R L C E N I A S H P K D A	470
CAG CTT TTC ATC CAG AAG AAA GCT GTG AAA AAC TTC ACT GAT GTC CAT CCT GAG TAT GGT	1503
Q L F I Q K K A V K N F T D V H P E Y G	490
GCC CAT ATT CAA GCT CTG CTG GAC AAG TAC AAT GGT GAA GCT GGC AAA CAG GAT GTG ATT	1563
A H I Q A L L D K Y N G E A G N Q D V I	510
AGA ACA TAT ACA CAA TCT ACA CCT CAT GTG TCT TCA AAG GAA AAA <u>TCT AAC</u> CTG TAA ACT	1623
R T Y T Q S T P H V S S K E K S N L	528
GTGTGTCACGTGCAGTTGGTCCATCCATGGATTTTACATCCACCTACCTGGAGAATAAGCTGTCAAAG	1691
ACATGAACGGATGTCCGCACAAGATAGAAAACGCTCTTCAGGCTTGATGCAGTAATGAAAAGTGGCTA	1799
TTCTTATATCTGCAATAGAAATTGATTGACTCTAATAACCTATTGCCTTCTAACACCACTGCTCCAGCC	1827
CTTTAATGATTTTATGCAGATACTTACGTCTAAAAAAGATTTTGTGTAGTTTGGAAATTAATCTCAA	1996
TACTTAAATGAGAAAAATGTGTTTCAAGTAGTTTGGGGTAAGAACCTAATTTCTGGATAAATTAC	1964
TGAAACAACCTACACTGTGATTTTTTTTTTTTCAACATAGGTGAACCTATTGGGAGTTTCATATTGA	2033
TAGAATGTAACCTGATTTTCAA <u>AATTAA</u> ATTGCCAGTATGTGTGATGTGATCATCTAGTTGGAAGC	2101
AATGTAACAGCAAGAAGTAAAGGAATCTATACATGTGCAGTACAAAAACAAAAA	2167
AAAAAA	2173

Figure 1 cDNA sequence and predicted amino acid sequence of turtle catalase. Numbers to the right refer to nucleotide and its deduced amino acid residues. The poly (A) signal (AATTAAA) is shown by underline. The predicted peroxisomal targeting signal is indicated by box, and two putative N-glycosylation sites by double underlines.

Table 1 Primer sequences used in this study.

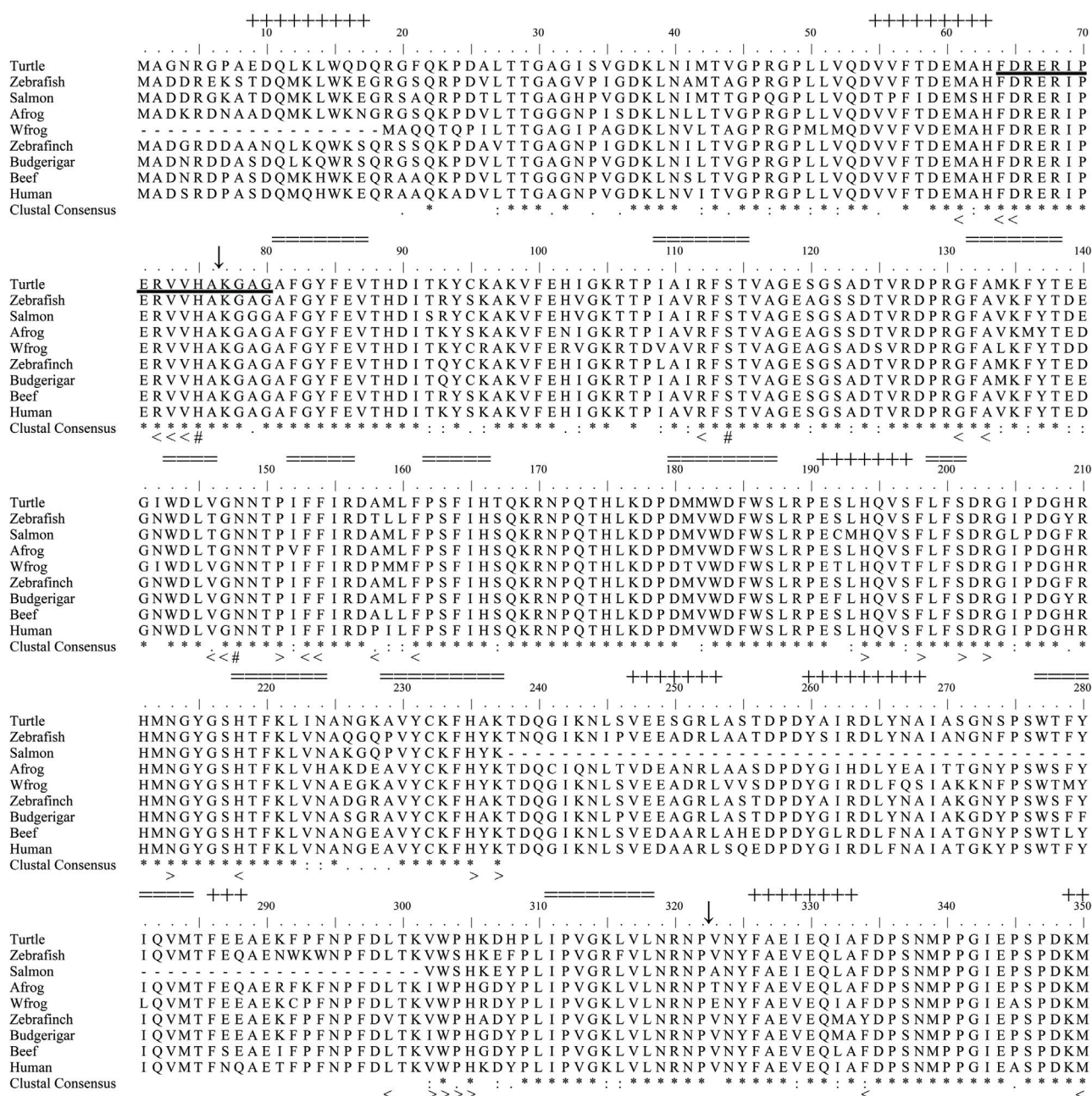
Primer	Sequence (5'-3')	Sequence information
C01F (forward)	TGACWGTGGKCCACGWGGWCCTCT	Degenerate primer for RT-PCR
C01R (reverse)	CTYTTYTGRCTATGGATRAAGGATG	
C02F (forward)	ATCCATAGYCARAARAGGAAYCCDCAG	Degenerate primer for RT-PCR
C02R (reverse)	GGCTRGGTTCAAYTCCTGGRGGCAT	
C03F (forward)	TGARGTGGAACAACCTGCCTTTGACCC	Degenerate primer for RT-PCR
C03R (reverse)	AGCRTTGTRYTTRTCCARRWGRGCTG	
5P1	AGGCTCCAGAAGTCCCACATCAT	5'-RACE primer
5P2	AAGAAGATGGGAGTGTTGTTGCC	5'-nested RACE primer
3P1	GAAGATGAACGCCAAAGACTGTGTGAG	3'-RACE primer
BCF (forward)	ATCCTGATACTACCGACACCGTT	Primer of CAT for semiquantative PCR
BCR (reverse)	ACCATTGTACTTGTCCAGCAGAGC	
BAF (forward)	TTGTCCCTGTATGCCTCTGGT	Primer of β -actin for semiquantative PCR
BAR (reverse)	TGGTTTCATGAATGCCACAGG	

**Figure 2** Phylogenetic tree showing the relationship of turtle catalase with the known catalases of vertebrate, with fruitfly catalase as outgroup. This tree was reconstructed by the neighbor-joining method with MEGA v5.0. Numbers at the nodes are bootstrap values representing their robustness (1000 replications). The scale bar indicates the estimated evolutionary distance between the groups. The GenBank accession numbers are indicated in the parenthesis. Turtle is shown by *.

The catalases of two animals selected from fish, amphibian, bird and mammal respectively were aligned with the turtle catalase as shown in Figure 3. The turtle catalase is divided into the four domains as described in the reviews by Nicholls *et al.* (2000) and Zámocký and Koller (1999). N-terminal arm contains the first 76 residues. Residues from 77 to 322 are β -barrel domain mainly comprising β -strands. Wrapping loop comprises residues from 323 to 432. The left residues are the α -helix domain containing four α -helices. Terminus N of N-terminal arm and α -helix domain are varied, while β -barrel domain is conserved.

The 3-D homology model of turtle catalase (Figure 4) was superimposed on the relative domains of bovine catalase structure (PDB ID: 3rgp) with QMEANScore of 0.627 for their identity (86%). The turtle catalase is homotetramer, and every subunit contains 15 α -helices and 9 β -strands, and binds one heme and one NADPH. β -barrel is composed of the former 8 β -strands. The residues responsible for secondary structure were demonstrated in Figure 3.

As shown in Figure 4, the active site heme is deeply buried within the core of subunit. The amino acids in contact with heme include Met61, Phe64, Asp65, Arg72,



(Continued Figure 3)

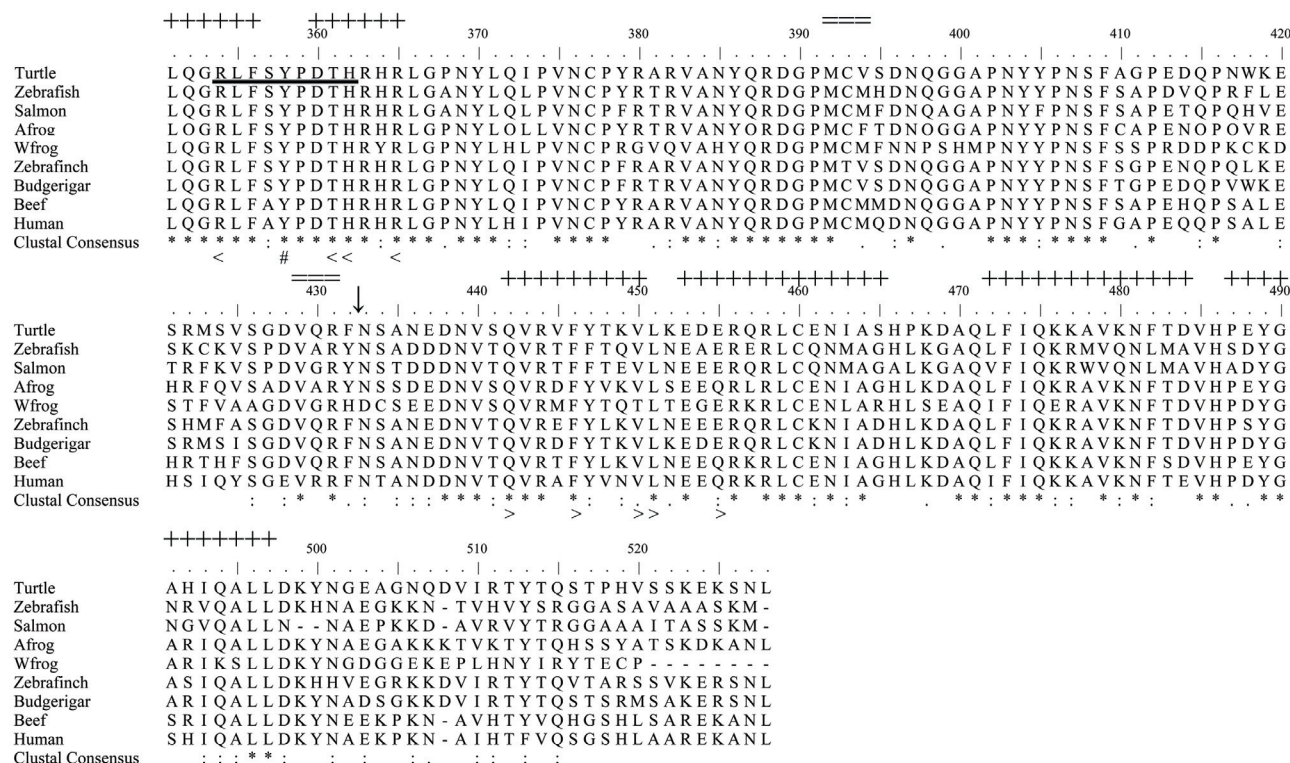


Figure 3 Alignment of amino acid sequences of catalases among the nine species by ClustalW 2.0. These animals include turtle, zebrafish (GenBank accession number NP_570987.1), Atlantic salmon (NP_001133774.1, Salmon), western clawed frog (NP_001011417.1, Wfrog), African clawed frog (ABK62836.1, Afrog), zebra finch (XP_002189160.1), budgerigar (AAO72713.1), beef (NP_001030463.1) and human (NP_001743.1). Different symbols in the line of Clustal consensus distinguish the different cases: an * indicates positions which have a single, fully conserved residue; a . indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix; and a . indicates conservation between groups of weakly similar properties - scoring = < 0.5 in the Gonnet PAM 250 matrix. Residues coordinating for secondary structure are shown on the first line: α helices are indicated by +, and β strands by =. The four main domains are separated by a down arrow on the second line. Different symbols below the line of Clustal consensus distinguish the different cases: substantial residues (His75, Ser114, Asn148 and Tyr358) are indicated by a #; residues in contact with heme a by a <; residues interacting with NADPH by a >. The proximal heme-ligand signature (residues 354–362) and the proximal active site signature (residues 64–80) are indicated by underline.

Val73, Val74, His75, Arg112, Ser114, Gly131, Ala133, Val146, Gly147, Asn148, Phe153, Phe154, Ala158, Phe161, His218, Leu299, Phe334, Met350, Arg354, Tyr358, Thr361, His362 and Arg365 (Figure 3). Among these residues, His75, Ser114, Asn148 and Tyr358 are regarded as the substantial residues (Nicholls *et al.*, 2000; Zámocký *et al.*, 2008). The proximal heme-ligand signature Arg-Leu-Phe-Ser-Tyr-Pro-Asp-Thr-His is located in the residue from 354 to 362, and the proximal active site signature Phe-Asp-Arg-Glu-Arg-Ile-Pro-Glu-Arg-Val-Val-His-Ala-Lys-Gly-Ala-Gly in the residue from 64 to 80.

NADPH is located in a cleft between the helical domain and β -barrel, and has no direct interaction with heme. The amino acids that appear to be important around NADPH include Pro151, His194, Phe198, Ser201, Arg203, Asn213, His235, Lys237, Val302, Trp303,

Pro304, His305, Gln442, Phe446, Val450, Leu451 and Glu455 (Figure 3). The amino acids contacting with heme or NADPH are completely identical in the catalases of turtle, beef, zebra finch and budgerigar, while one or two conservative substitutions happen in human, fish and frogs. Turtle catalase belongs to the small-subunit group with bovine and human catalases, due to conservation of amino acids binding NADPH and the lack of flavodoxinlike structure at the extended carboxyl ends.

Figure 5 is the electrophoresis photo of semiquantitative PCR. The mRNA expressions of the catalases in liver, brain, kidney and muscle were higher than those in lung, gut, heart and spleen. The catalase activity in liver was obviously higher than those in the rest tissues ($P < 0.05$) as shown in Figure 6. No differences were observed among enzyme activities of the rest tissues.

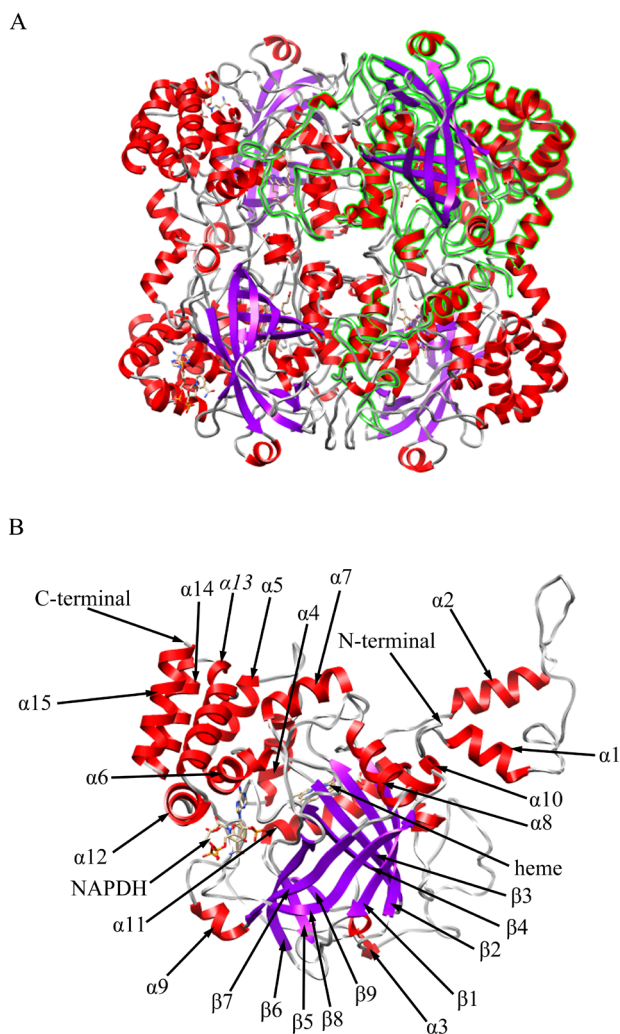


Figure 4 The 3-D homology model of turtle catalase. The model was created by SWISS-MODEL and then was superimposed to bovine catalase structure (PDB ID: 3rgp) by the Chimera 1.6.2. The α -helix is shown in red, and β -strand in violet. A: One subunit of homotetrameric turtle catalase is indicated by green line; and B: One subunit contains one heme and one NADPH, and comprises 15 α -helices and 9 β -strands.

4. Discussion

Catalase can efficiently degrade H_2O_2 to keep redox homostasis in cells (Scandalios, 2005). The mRNA expression and enzyme activity will change due to environmental stress (Lushchak, 2011), and catalase also participates in activation of immune cells, inflammation, cellular propagation and apoptosis by regulating concentration of H_2O_2 (Goyal and Basak, 2010; Scandalios, 2005). The conserved function of catalase among aerobic organisms is assured by high conservation of amino acid sequence and structure. The turtle catalase has higher identity and similarity with the catalases of other vertebrates. The predicted size of the polypeptide

59.80 kDa is very close to the catalases from human (59.62 kDa), pig (57.18 kDa) and bovine (57.58 kDa).

The CA repeats in the catalases of mouse (Reimer and Singh, 1996), rat (Clerch, 1995) and zebrafish (Gerhard *et al.*, 2000) are predicted to form a stem-loop structure, and therefore may be a common cis-acting regulatory element. The absence of a repeat sequence in turtle catalase might suggest some dissimilarity in regulating gene expression between turtle and these three animals. PST1 in turtle catalase Ser-Asn-Leu is different from the PST1 having been found in rat and mouse (Ala-Asn-Leu) (Trelease *et al.*, 1996) and human (Lys-Ala-Asn-Leu) (Purdue and Lazarow, 1996). Though the conserved sequence of PST1 is a tripeptide with a small neutral amino acid at the first position, an amino acid residue capable of hydrogen bonding in the middle and a hydrophobic amino acid at the carbon terminus, it is still species-specific (Brocard and Hartig, 2006).

His75, Ser114, Asn148 and Tyr358 are regarded as essential residues in typical catalases. His75 is situated in active site, allowing the proper binding and reduction of a peroxide molecule, while Tyr358 has direct contact with heme (Nicholls *et al.*, 2000; Zámocký and Koller, 1999). Both Ser114 and Asn148 are located in β -barrel domain. Ser114 stabilizes the environment around heme, while Asn148 makes the entry to the active site more polar (Zámocký *et al.*, 2008; Zámocký and Koller, 1999). Tyr132 has been recently determined to be also critical for catalysis (Jakopitsch *et al.*, 2003). Furthermore, the conservation of amino acids contacting with heme or NADPH suggests the important function of the two cofactors. Heme is a key part in catalytic proceeding (Nicholls *et al.*, 2000; Zámocký and Koller, 1999). Though the function of NADPH is still under discussion, NADPH has been proposed to play critical role in catalysis as an electron source (Goyal and Basak, 2010; Nicholls *et al.*, 2000; Zámocký and Koller, 1999).

The relatively higher activity and mRNA expression of catalase in liver of adult turtles is similarly observed in fish (Ansaldi *et al.*, 2000; Bagnyukova *et al.*, 2005; Filho *et al.*, 1993; Kim *et al.*, 2010; Stratulat *et al.*, 2010; Trenzado *et al.*, 2006), amphibians (Dadras *et al.*, 1996) and mammals (Goyal and Basak, 2010; Meng and Zhang, 2003; Sohal *et al.*, 1995). Vertebrate liver exhibits high metabolism and oxygen consumption, and it probably produces a great number of ROS (Lattuca *et al.*, 2009; Trenzado *et al.*, 2006). Moreover, liver is an important organ for xenobiotic detoxification and considered to be powerful ROS generator (Bagnyukova *et al.*, 2005; Lushchak *et al.*, 2001).

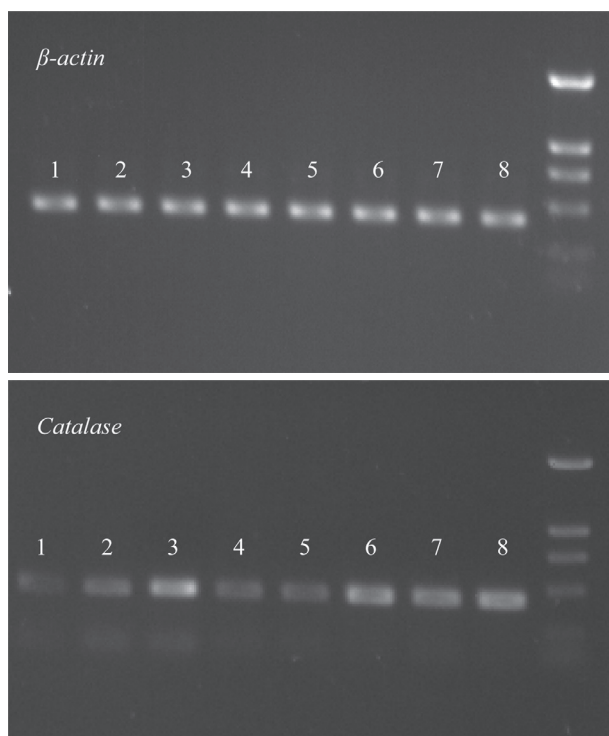


Figure 5 Results of semiquantitative PCR in a 1% agarose gel. Two panels show the results of reference β -actin and catalase. In both panels, Lanes 1 to 8 are for lung, gut, muscle, heart, spleen, kidney, brain and liver, respectively; and the last lane for molecular standard 2-kb DNA ladder.

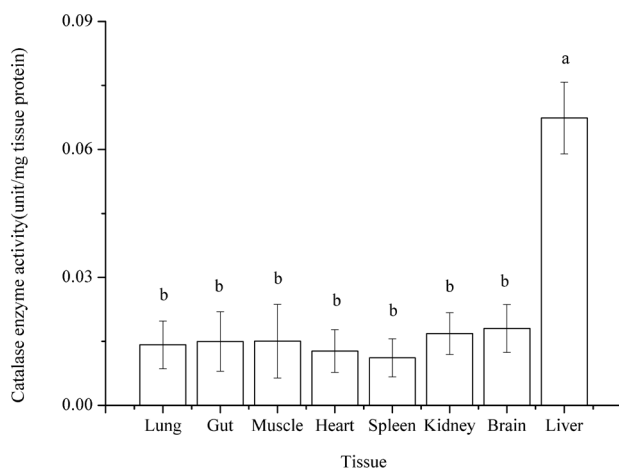


Figure 6 CAT activities of eight tissues in turtles (Mean $\pm$ S.D., $n = 8$). Values with different letters are significantly different ($P < 0.05$) from each other.

The mRNA expression of catalases in the brain, kidney and muscle are higher than those in lung, gut, heart and spleen, though no difference exists in catalase activity. However, in the studies of grass carp (*Ctenophorus pictus*) (Stratulat *et al.*, 2010), marine teleosts (Filho *et al.*, 1993), Antarctic fishes (Ansaldò *et al.*, 2000) and mammals (Goyal and Basak, 2010; Sohal *et al.*, 1995) the enzyme

activities in kidney have been found to be medium, and those in brain or muscle are lower. A moderate level of catalase activities in heart has been observed in Mongolian gerbil (*Meriones unguiculatus*) (Sohal *et al.*, 1995), marine teleosts (Filho *et al.*, 1993) and Antarctic fishes (Ansaldò *et al.*, 2000), but the heart of sturgeon (*Acipenser naccarii*) and trout (*Oncorhynchus mykiss*) (Trenzado *et al.*, 2006) exhibit a low level. Moreover, mRNA expression was detected in the frog by Northern blotting with the results from high to low, liver > kidney > lung > intestine > heart > skeletal muscle (Dadras *et al.*, 1996). These observations imply that the investigated vertebrates would have species-specific tissue patterns of mRNA expression and activity according to their environments.

The presented research provides novel molecular information about the turtle's catalase gene. The findings may contribute a new way to evaluate the function of catalase and its control mechanism at the molecular level for the soft-shelled turtle.

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